Thesis for doctoral degree (Ph.D.) 2013

Studies on Bone and Mineral Disorders In Chronic Kidney Disease: Determinants and Survival Implications **Ting Jia**

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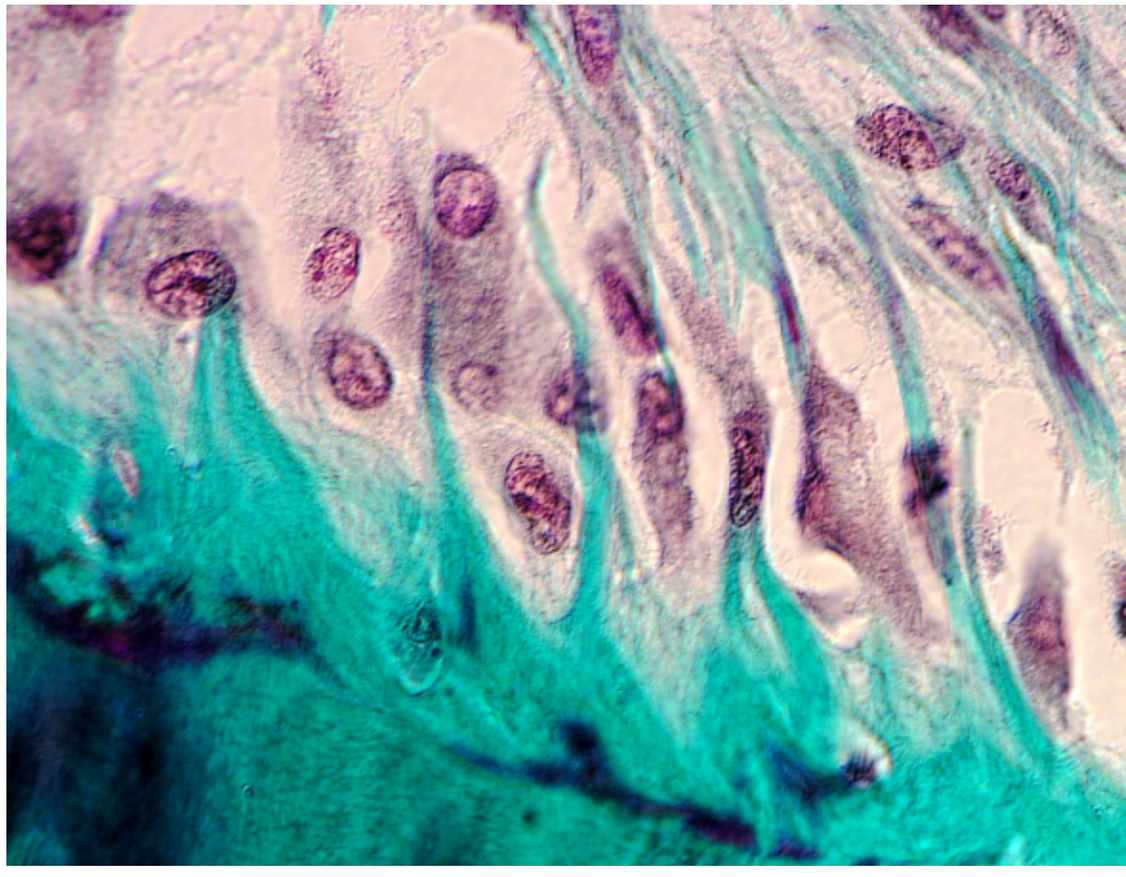

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Studies On Bone and Mineral Disorders in Chronic Kidney Disease: Determinants and Survival Implications

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A thesis submitted for the degree of

Doctor of Philosophy

Sweden 2013

Typeset in LaTeX 2$_\varepsilon$ by author.
Front cover shows osteoblast cells (Photo by Dr.Juan Antonio Bravo Soto).
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ISBN [978-91-7549-325-1]

I would like to dedicate this thesis to my parents.
谨将此文献给我挚爱的父母

Abstract

Disruptions in mineral metabolism occur already at an early stage of chronic kidney disease (CKD) and may eventually lead to a complex of CKD-mineral and bone disorders (CKD-MBD) characterized by alterations of mass, turnover rate, mineralization and strength of bones, and high risk of vascular calcification, fractures and other complications resulting in worse survival. The aim of this thesis is to increase the understanding of some aspects of CKD-MBD with special emphasis on clinical and laboratory determinants and implications for clinical outcome.

In **Study 1** we defined and tested a novel non-surgical, adjustable model of tubulointerstitial nephropathy in mice by adding adenine to a casein-based diet and showed that this model could induce and maintain a uremic phenotype with decline of renal function and initiation of alterations linked to CKD-MBD.

In **Study 2** we investigated the impact of classical risk factors for osteoporosis such as age, body composition, and nutritional status in end-stage renal disease (ESRD) subjects, and how these factors influence the relationship between bone mineral density (BMD) and mortality in patients with ESRD. We found that low BMD is associated with low total fat mass, poor nutritional status and increased mortality risk in ESRD patients.

In **Study 3** we studied determinants of the variability of two bone metabolic markers, fibroblast growth factor-23 (FGF23) and intact parathyroid hormone (PTH) in ESRD patients treated by peritoneal dialysis, hemodialysis or online hemodiafiltration. We found that baseline vitamin D status and serum phosphorous were independent determinants of the longitudinal variation in PTH and FGF23, respectively and that the intra-subject variability of FGF23 was lower than the variability of PTH irrespective of dialysis mode.

In **Study 4** we analyzed baseline serum insulin-like growth factor 1(IGF-1) concentration and the longitudinal changes of IGF-1 over 1-year in CKD stage 5 patients starting on dialysis in relation to bone and mineral metabolism parameters including BMD, nutritional status, and mortality. We found that IGF-1 associates with markers of mineral and bone metabolism and that IGF-1 is a strong independent predictor of mortality risk in CKD stage 5 patients.

List of Publications

1. **Jia T*** ,Olauson H* , Lindberg K, Amin R, Edvardsson K, Lindholm B, Andersson G, Wernerson A, Sabbagh Y, Schiavi S, and Larsson TE. A novel model of adenine-induced tubulointersititial nephropathy in mice.BMC Nephrol. 2013 May 30;14(1):116. * Shared first authors

2. Park SH* , **Jia T***, Qureshi AR, Bárány P, Heimbürger O, Larsson TE, Axelsson J, Stenvinkel P, and Lindholm B. Determinants and survival implications of low bone mineral density in end-stage renal disease patients. Journal of Nephrology, 2012, 26(3 (May-June 2013)): 485-494. * Shared first authors

3. **Jia T**, Qureshi AR, Brandenburg V, Ketteler M, Bárány P, Heimbürger O, Uhlin F, Magnusson P, Fernström A, Lindholm B, Stenvinkel P, and Larsson TE. Determinants of fibroblast growth factor-23 and parathyroid hormone variability in dialysis patients. Am J Nephrol. 2013; 37 (5):462-71.

4. **Jia T**, Gama-Axelsson T, Heimbürger O, Bárány P, Lindholm B, Stenvinkel P, and Qureshi AR. Insulin-like growth factor-1 and survival in end-stage renal disease patients. Clin J Am Soc Nephrol. 2013 Oct 31. [Epub ahead of print].

List of Abbreviations

ANCOVA	Repeated measures analysis of covariance
APD	Automated peritoneal dialysis
B-ALP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
CAPD	Continuous ambulatory peritoneal dialysis
CI	Confidence intervals
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease-mineral and bone disorders
CTX	C-telopeptide cross laps
CV	Coefficient of variation
CVD	Cardiovascular disease
DEXA	Dual-energy X-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
ESRD	End-stage renal disease
FGF23	Fibroblast growth factor 23
GFR	Glomerular filtration rate
GWAS	Gene-based genome-wide association studies
HD	Hemodialysis
HDF	Hemodiafiltration
HDL	High-density lipoprotein
HR-MRI	High resolution magnetic resonance imaging
HR-pQCT	High resolution peripheral quantitative computed tomography
ICAM-1	Intercellular adhesion molecule
ICC	Intra-class correlation
ICTP	Carboxy-terminal telopeptide of type I collagen
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin -6
MIA	Malnutrition, Inflammation, and Atherosclerosis
MIMICK-2	Mapping of Inflammation Markers In Chronic Kidney Disease-2 study in peritoneal dialysis patients
OPG	Osteoprotegerin
OPN	Osteopontin
PD	Peritoneal dialysis
PEW	Protein-energy wasting
PICP	Type I collagen
PTH	Parathyroid hormone
QCT	Quantitative computed tomography
RANKL	Receptor activator of nuclear factor-kappa B ligand
ROC	Receiver operator characteristics
SD	Standard deviation
SGA	Subjective global assessment
TNF	Tumor necrosis factor
TRAP	Tartrate-resistance acid phosphatase
TSH	Thyroid stimulating hormone
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell

Contents

Chapter 1

INTRODUCTION

1.1 BONE MINERAL DISORDERS IN CKD

Chronic kidney disease (CKD) is associated with numerous metabolic and nutritional alterations affecting among others mineral metabolism and bone health which are interrelated and together form an entity called CKD-mineral and bone disorders (CKD-MBD). CKD-MBD is associated with disturbances of phosphate and calcium homeostasis as well as with changes in key regulators of bone status such as parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). These alterations may lead to renal osteodystrophy with bone loss, osteoporosis, and potentially fractures, and increased risk for premature vascular calcification, adding significantly to other common causes of cardiovascular disease (CVD), the leading cause of death in CKD patients. CKD-MBD is thus thought to be a major contributor to the high mortality among patients with CKD [1].

While many of the circulating mediators of CKD-MBD can be relatively easily measured, a detailed assessment of bone status requires more complex methods some of which such as bone biopsy are not readily available. However, the measurement of bone mineral density (BMD) usually performed by dual-energy X-ray absorptiometry (DEXA) is a more convenient method in the clinical setting and is increasingly regarded as an integral component of assessment of bone mass, presence and extent of osteoporosis, and risk of fractures. BMD is a well-established key parameter for monitoring bone disease in CKD patients, although it should be noted that, there are many common factors affecting BMD not specifically related to CKD-MBD, such as age, gender, menopause, estrogen consumption, body mass, cigarette smoking, alco-

hol abuse, excess glucocorticoid exposure, physical activity and genetic factors [2]. It should also be noted that BMD cannot fully describe the status of bone fragility since bone status is due to many dynamic factors such as abnormal bone turnover and remodeling, leading to impairment of bone micro-architecture.

Disruption in mineral metabolism occur already at early stages of CKD, leading as the disease progresses to alterations in bone mass, bone turnover, mineralization and bone health [3]. Disorders of bone structure and bone mass may result in severe osteoporosis and marked risk of fractures. Moreover, and even more important, because of the close links between bone status and soft tissue calcification, these alterations associate with vascular calcification, sometimes described as vascular ossification, leading to clinically manifest CVD and increased mortality. The following brief review which in part is based on a recent review article from our group [4] summarizes the current understanding of causes of CKD-MBD, and its consequences for clinical outcome in CKD patients.

1.2 CAUSES OF IMPAIRED BMD IN CKD

1.2.1 Renal function, body weight and age

BMD decreases progressively in patients during the course of progression from mild to severe degrees of renal failure and the decrease in BMD is thus most pronounced in patients with the lowest glomerular filtration rate (GFR) [5]. Bianchi *et al.* reported that patients with pre-dialysis renal failure have reduced BMD which correlated to the reduction of renal function [6]. In end-stage renal disease (ESRD), the reported prevalence of bone mineral deficiency is higher but variable depending both on population-specific characteristics and on the techniques and body sites used for measurements. The effects of renal replacement therapy on bone mass vary between the different therapies. Although renal transplantation results in improvement of many aspects of CKD-MBD, it may further worsen bone mineral deficiency. BMD measured in the lumbar bone decreased significantly after six months of transplantation [7], which seemed to be mediated primarily by glucocorticoid usage after transplantation [8]. On the other hand, it was reported that in patients on dialysis treatment, BMD did not change during the first year of dialysis treatment, neither in patients on hemodialysis (HD) nor in patients treated by peritoneal dialysis (PD) [9]. Whereas an increased risk of hip fracture was

found to be related to the duration of dialysis [10], the impact of dialysis treatment as such on BMD is still not clear.

There is a positive correlation between body weight and BMD in the general population [11]. Similarly, several studies demonstrated that the body size relates with BMD also in CKD and ESRD patients [12; 13; 14]. In general body mass index (BMI) associates with BMD or bone mass as measured by DEXA and BMI is a predictor of BMD also in ESRD patients [5]. Thus, not surprisingly, body weight and BMI were primary responsible factors for BMD variation, osteoporosis and fractures [2; 15], and were determinants also of the Z-score of BMD at mid-radius, femoral neck, lumbar spine and measurement sites, and total body BMD, in HD patients [16]. It should be noted however that the relationship between BMI and BMD in the lumbar spine area is confounded by presence of aortic calcification leading to erroneously high measurements of BMD. Obesity also is associated with elevated BMD of weight-bearing bones and ribs, suggesting that obesity *per se* or indirectly via the increase of body mass may prevent bone loss [17]. On the other hand, reduced lean body mass and sarcopenia associated with bone loss as demonstrated in in elderly people [18], putatively due to reduced impact on bone remodeling which through increased mechanical load forces of lean tissue may serve to strengthen bone [19]. It is not surprising that a large body mass is closely related with high BMD also in CKD patients [20] and body mass was reported to correlate with BMD at all body sites in HD patients [16].

The association of body fat mass with bone status may in addition be related to the metabolic activity of adipose tissue potentially affecting the skeleton as well as by hormonal alterations linked at least in part to the amount of adipose tissue, such as alterations in vitamin D metabolism, peripheral estrogen production [21], free fraction of sex steroids [22], insulin levels, and leptin levels [23].

Appropriate regular physical activity and especially weight-bearing exercise could be recommended as measures aiming at maintaining muscle mass and muscle strength and thereby improving bone quality and possibly reducing fracture risk in the general population [24; 25] and presumably such measures are important also among CKD patients. As we grow older, bone mass decreases and bone loss is thus an age dependent process [26]. Age-related bone loss occurs especially if there is a decline of physical activity, as observed also experimentally in mice [27], and in humans the age-related bone loss occurs in adults at a rate of 1-2% after the age of 40 years [28]. Dialysis

patients, who in general are elderly and often physically inactive, have an increased risk of low trauma fractures [2]. However, in dialysis patients, as mentioned above, many other factors could weaken the relationship between age and BMD. For example, in one study, age was inversely correlated with BMD in female, but not in male HD patients [29], whereas in another study, age showed a robust negative correlation with femoral neck BMD in PD patients [14].

In CKD patients, the fine-tuned regulation of BMD is complicated by the abnormal metabolism of calcium, phosphorus, PTH, vitamin D and other metabolites and hormones [30]. Serum concentrations of intact PTH, ionized calcium and phosphate, also alkaline phosphatase and vitamin D, are well-established commonly used markers of CKD-MBD that are thought to reflect or at least be related to bone status and possibly bone turnover and bone formation [31].

In dialysis patients, the dialysis procedure may involve factors that can influence CKD-MBD. For example, a low calcium concentration in the dialysate may promote bone loss by stimulating the PTH secretion, and in one study, PD patients on low-calcium PD solutions tended to have a low BMD [32]. On the other hand, a meta-analysis in patients undergoing long or long-frequent HD showed that a dialysate with calcium concentration greater than 1.5 mmol/L could prevent an increase in PTH and decline in BMD without increasing the risk of vascular calcification [33]. Thus, a high calcium dialysis solution may protect against osteoporosis [14] whereas a low calcium dialysate may increase the risk of osteoporosis and hyperparathyroidism [34] especially during long-term use [35]. Some studies found an inverse correlation between PTH levels and mid-radius BMD reflected by Z-scores in HD patients [36; 37]. Others could not find such correlation of PTH and BMD in dialysis patients [38]. These discrepancies may be due to differences in follow-up time. While BMD mainly reflects long-term influence of CKD-MBD factors [39], PTH has more immediate effects on the metabolic state of bone in CKD patients and an increased PTH level will probably not result in substantial bone loss in the short-term. Another factor of importance is vitamin D which was reported to be linked to BMD, but not to fracture incidence, in patients with CKD stage 3 to 5 patients [40; 41]. Vitamin D supplementation reduces serum PTH levels and improves bone strength in animal studies [42].

1.2.2 Fibroblast growth factor 23 and osteoprotegerin pathway

Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone that inhibits the calcitriol synthesis and affects bone turnover rate [43]. Moreover, FGF23 is an important inhibitor of PTH secretion [44]. In a large community-based cohort study, higher FGF23 concentrations were weakly associated with greater lumbar spine BMD and total hip BMD [45]. The FGF23 concentration is elevated in parallel with declining renal function and this elevation occurs earlier than that observed for phosphate. There are conflicting data about the relationship between FGF23 and BMD in CKD and dialysis patients, some studies have documented such a relation [46; 47] whereas in other studies no such association was found [48]. These discrepancies at least in part be related to the particular sites at which BMD was measured, and to the type of instrument (DEXA or computed tomography) that was being used. It should be noted that as the influence of FGF23 on bone mineralization could mainly be indirect, i.e. an effect of the degree of hypophosphatemia caused by FGF23, rather than reflecting a direct effect of FGF23 on bone [49], the relation between FGF23 and BMD could be confounded by numerous factors such as GFR, nutritional intake and concurrent treatment with phosphate binders.

The osteoprotegerin (OPG) and the receptor activator of nuclear factor-kappa B ligand (RANK/RANKL) systems play an important roles in the regulation of osteoclast formation, activity, and survival in normal and pathological states of bone remodeling. Biochemical markers, such as C-telopeptide crosslaps (CTX) and bone-specific alkaline phosphatase (B-ALP) are markers of bone resorption and bone formation that have been used for prediction of fracture risk, independent of other methods for monitoring osteoporosis, such as BMD [50]. The negative regulation of osteoclastic bone resorption exerted by OPG could increase BMD and bone volume by decreasing the active osteoclasts as demonstrated by in vitro studies [51]. OPG levels increase with increasing age; this age-dependent increase in OPG might be a counter-regulatory mechanism preventing further bone loss in elderly subjects. OPG, and also FGF23, associate with myocardial damage and aortic pulse wave velocity in CKD patients, thereby linking CKD-BMD with CVD [52]. OPG is linked to osteoporosis, and loss of muscle mass as well as of fat mass [53]. There is a positive relationship between OPG and femoral neck BMD in HD patients indicating that OPG perhaps could be used as an initial screening tool of bone loss and presence of CKD-MBD in ESRD patients [54; 55]. Apart from being a bone biomarker, OPG associates with severity of coronary calcification in non-dialysis CKD patients [56]. Unlike OPG, the free and total RANKL levels de-

crease with age, possibly due to a general age-related reduction of cell activity [57]. The circulating concentration of OPG increases independently of the changes of serum PTH in uremic patients [58]. In pre-dialysis CKD stage 1-5 patients, serum RANKL negatively, and OPG positively, were found to be associated with femoral neck BMD [59]. OPG and adipose tissue derived leptin associated with osteoporosis in patients with chronic obstructive pulmonary disease [53] suggesting that links between these markers and loss of bone mass could be influenced by fat mass.

1.2.3 Wasting and bone loss

The results in the literature describing the relationship between skeletal muscle and bone loss are equivocal. Some studies demonstrated a clear association between bone mass and lean tissue [60; 61], but others could not confirm this [62; 63]. Osteoporosis is associated with sarcopenia in elderly populations [18], putatively due to an impact on bone remodeling through increased mechanical load forces of lean body mass. Insulin-like growth factor 1(IGF-1) is a component of the IGF-1/growth hormone system and is also an important regulator of bone growth and an early marker for low bone mass in pre- and post-menopausal women [64]. A recent study showed that the IGF-1/Akt pathway is involved in osteoporosis-related muscle atrophy, suggesting that BMD may serve as a nutrition marker reflecting muscle atrophy associated with osteoporosis [65]. In CKD and ESRD, only a few studies have so far demonstrated a positive relationship between IGF-1 concentration and BMD [66]. However, it is well established that nutritional status and bone status are closely linked; appropriate regular physical activity, especially weight-bearing exercise, is needed to maintain muscle mass and muscle strength, and associate with improved bone quality and reduction of fracture risk [67].

Anti-osteoporosis treatment with bisphosphonates as a strategy for preventing fractures in CKD patients also may reduce the progression of extra-osseous calcification and inhibit the development of atherosclerosis [68]. However, in advanced stages of CKD, bisphosphonates due to the risk of side-effects should be used with caution in carefully selected patients [69]. Whereas vitamin D supplementation has not been demonstrated to decrease the fracture incidence in patients with ESRD, animal studies show that vitamin D may reduce serum PTH levels and improve bone strength [70]. Vitamin D supplementation improves various biochemical endpoints linked to bone status [71], suggesting that vitamin D might improve BMD in CKD patients. Nevertheless, the use-

fulness and safety of vitamin D supplementation in ESRD patients is still not clear [72].

1.2.4 Biomarkers of bone formation

Several other biochemical markers of bone turnover may be considered for the diagnosis and monitoring of bone metabolic disease [73]. Recent studies have implicated the skeleton in energy metabolism as well as suggested that adipose tissue derived peptides i.e., adipokines, can affect the function of osteoclasts. Adiponectin, an anti-inflammatory, anti-atherogenic adipokine, has recently been shown having a negative effect on bone formation by stimulating receptor activator of NF-κB ligand for osteoclastogenesis, thus inducing bone resorption. A high concentration of adiponectin is observed in CKD patients [74], and has been found to be inversely related to BMD in HD patients [75; 76]. Since adiponectin is negatively associated with BMD in the general population, this could be in line with a link between hyper-adiponectinemia and poor outcomes. A link between leptin, the protein product of the obesity gene in fat tissue, and BMD has been hypothesized but this association is equivocal. Besides these markers mentioned above, osteocalcin, carboxy-terminal propeptide of type I collagen (PICP) and B-ALP may reflect bone formation whereas carboxy-terminal telopeptide of type I collagen (ICTP) reflect bone resorption. Some studies have demonstrated correlations between some of these bone markers and histomorphometric parameters reflecting bone status in CKD and ESRD patients [77; 78].

1.3 CONSEQUENCES OF BONE LOSS IN CKD

1.3.1 Vascular calcification

It is well-established that vascular calcification/ossification is closely linked to CKD-MBD via various mechanisms including the inability of bone, and especially adynamic bone, to absorb the excessive amounts of circulating minerals such as calcium and phosphate accumulating in patients with renal insufficiency. Evidence also suggests that vascular calcification is linked to bone-related proteins, such as B-ALP, osteocalcin, osteopontin (OPN), and Runx2; these proteins are expressed in the calcified vascular lesions. Watanabe *et al.* [79] found a close relationship between coronary artery calcification and bone loss in non-dialyzed CKD patients, suggesting that impaired bone formation could accelerate the progress of coronary artery calcification.

Increasing evidence suggests that the RANK/RANKL/OPG pathway, a key regulator of bone formation, may be involved in vascular calcification. Osteoblasts and active T cells synthesize RANKL, and RANK is expressed in osteoclasts, endothelial cells and vascular smooth muscle cell (VSMC). Previous studies demonstrated that the RANKL system is related to cardiovascular events and coronary artery calcification [80; 81]. The RANK/RANKL directly promotes VSMC calcification [82] through activating the NF$-\kappa$B pathway and release of tumor necrosis factor (TNF) and interleukin (IL)-6 while OPG inhibits the process leading to vascular calcification [83]. Osteoclast-like cells express tartrate-resistance acid phosphatase (TRAP) in calcified lesions, and the RANK/RANKL directly stimulates TRAP positive osteoclast-like cell formation, suggesting that osteoclasts might promote vascular calcification [84]. Some osteoporosis therapies, such as bisphosphonates (pyrophosphate analogs), denosumab (a monoclonal inhibitor of RANKL) and a recombinant fusion protein of OPG may inhibit vascular calcification [85]. CKD, especially ESRD is associated with elevated circulating FGF23 levels and reduced Klotho activity; FGF23 (and 1, 25-dihydroxyvitamin D) influences post-transplant bone mineral loss while Klotho protein deficiency contributes to accelerated aging with arterial calcification and osteoporosis [86; 87].

1.3.2 Osteoporosis

Osteoporosis is a skeletal disorder characterized by loss of bone strength and microarchitectural deterioration of bone tissue, leading to increased risk of fractures - the main clinical manifestation of osteoporosis [88]. In ESRD patients, osteoporosis is a part of a broader field of metabolic bone problems named uremic osteodystrophy. In dialysis patients, the reported prevalence of bone mineral deficiency varies depending on the techniques of measurements. The diagnosis of osteoporosis usually depends on reduction in bone mineral content reflected by reduced BMD as assessed by DEXA. The prevalence of osteoporosis in HD patients was reported to range from 14% to 50% depending on the site in the skeleton where it was studied [16; 89; 90]. In PD patients, BMD was not significantly different from age- and sex-matched reference population data [91] while in another study BMD was higher than in HD patients [92]. However, there is an increased prevalence of vertebral and hip fractures among CKD patients as a group compared with the general population in all age groups, and there are links between osteoporosis increased mortality, possibly reflecting links of low BMD with poor nutritional status and vascular calcification [93].

Low BMD is a predictor of the risk of fracture which is a significant consequence of bone loss in the CKD population. The World Health Organization (WHO) uses the T-score of BMD measured in the spine and hips as an index for the classification of osteoporosis and osteopenia. In a previous report, total T-score correlated with T-scores at different body sites, especially T-score from the forearm which could be a promising site for BMD measurement also in CKD patients [94]. The incidence of hip fractures in CKD stage 5 patients is 17 times higher than in the general population in the United States, one reason being the high prevalence of osteopenia and osteoporosis in these patients. The use of BMD as a diagnostic tool for osteoporosis and fracture risk in CKD patients is controversial. Jamal *et al.* [95] failed to find a correlation between BMD measured by DEXA and fractures in 104 elderly HD patients whereas Atsumi *et al.* [89] found that lumbar spine BMD associated with vertebral fractures in HD patients. Other studies have also suggested a weak predictive value of BMD for fractures in dialysis patients [14; 16]. It is not clear whether peripheral or central bone, or cortical or trabecular bone, are to be preferred for the evaluation of BMD in CKD patients.

Bisphosphonate treatment to improve BMD and lower the fracture risk is common in several populations and the usage of this and other types of anti-osteoporosis medications has been reported in CKD populations. One study shows that a low dose of alendronate therapy could protect against bone loss at Ward's triangle in hip in HD patients [96]. However, the risk of side-effects of anti-osteoporosis treatment is not insignificant, and this has reduced its wider clinical use among CKD patients.

Recently, some researchers underlined that the standard WHO classification of osteoporosis or osteopenia to classify bone status in CKD stage 4 or 5 patients may not be appropriate. As mentioned above, a possible explanation is that BMD determined by DEXA may be falsely elevated in CKD patients due to sclerosis of posterior elements and calcification of large arteries including aorta [97]. DeVita *et al.* concluded that the BMD measured by DEXA was a poor indicator of renal osteodystrophy in HD patients [78] and Malluche and Faugere found that DEXA could not discriminate between different types of renal osteodystrophy [98]. While DEXA may not be an ideal tool for assessing bone quality and bone strength [99], bone biopsy, followed by histomorphometric analysis, provides qualitative and quantitative information about bone remodeling and bone status, in particular regarding the micro-architectural changes in bone tissue. However, this invasive approach is usually not available for the evalua-

tion of osteoporosis in CKD patients in a clinical setting [100]. Instead, alternative non-invasive imaging techniques for assessment of bone health, such as quantitative computed tomography (QCT), high resolution magnetic resonance imaging (HR-MRI) and high resolution peripheral quantitative computed tomography (HR-pQCT) are increasing being used in clinical studies [101].

1.3.3 High Mortality

Bone disorders, including the loss of bone mass, are related with high mortality in the general population. The Third National Health and Nutrition Examination Survey (NHANES *III*) reported that subjects in the lowest quartile of BMD had a greater risk of death than those within the highest quartile of BMD [102]. In one study there was a significant association between BMD and non-trauma mortality in women [103]. Another community-based study from Europe confirmed that BMD is a strong predictor of subsequent mortality in both women and men [104]. In CKD patients, studies including one on PD patients [105] have shown links between BMD and survival.

In the general population, BMD can be considered to be a general marker of health and aging, and as there are many factors associated with these general characteristics such as physical activity, and mental and social status, the relationship between low BMD and mortality is probably not a direct causal one. on the other hand, among CKD patients, the association between bone loss and high mortality could be due also to more specific and even causal relations such as links between low BMD and vascular calcification, or between BMD and other conditions that increase the risk for CVD events in CKD patients such as poor nutritional status and inflammation.

The mortality predictive role of BMD in patients with CKD is thus influenced by numerous factors such as nutritional status. For example, in ESRD patients, a low BMD correlates with protein-energy wasting and CVD, and in our studies a low value of BMD was found to be an independent predictor of all-cause and cardiovascular mortality [66]. One explanation for the increased mortality in patients with low BMD may be that this group suffers to a larger extent from adynamic bone disease, a condition in which the skeleton is incapable of buffering excess calcium and phosphate, leading to more extensive vascular calcification and as a consequence increased CVD mortality. In postmenopausal women, hormonal deficiency plays an important role in reducing BMD

[106; 107], and estrogen deficiency, as well as calcium balance, at menopause could play an important role and possibly explain at least in part the connection between low bone mass and mortality also in CKD patients [108].

Chapter 2

AIM

The overall aim of this thesis is to enhance the understanding of determinants and clinical implications of bone and mineral disorders in chronic kidney disease.

Specifically, the objectives of current thesis were:

- To develop a non-surgical adenine-induced renal failure model in mice and to explore its potentials for the study of bone and mineral disorders in CKD **(Study 1)**

- To investigate determinants of bone mineral density and its implications for the clinical outcome of ESRD patients **(Study 2)**

- To evaluate the determinants of intra-subject variability of the traditional bone metabolism marker (PTH) and a more novel marker (FGF23) in a cohort of peritoneal dialysis (PD) patients and to perform a comparative analysis of PTH and FGF23 variability in patients receiving PD, hemodialysis (HD) or online hemodiafiltration (HDF) **(Study 3)**

- To investigate links between IGF-1 and bone mineral metabolism parameters and the mortality predictive role of IGF-1 and its changes following initiation of dialysis treatment in CKD stage 5 patients **(Study 4)**

Overall design of the current thesis

Figure 2.1 shows the overall design of the current thesis with links among the four included studies. **Study 1** demonstrated that bone mineral disorders phenotypes could be induced in an adenine-induced renal disease model in mouse, **Study 2** reported on determinants of BMD and association of low BMD with wasting and mortality in CKD stage 5 patients, **Study 3** explored determinants of the longitudinal variation of bone mineral biomarkers PTH and FGF23 and their variability in patients undergoing peritoneal dialysis, hemodialysis and online hemodiafiltration, and **Study 4** showed that low serum IGF-1 associates with body composition and markers of mineral and bone metabolism, and predicts increased mortality risk in incident dialysis patients.

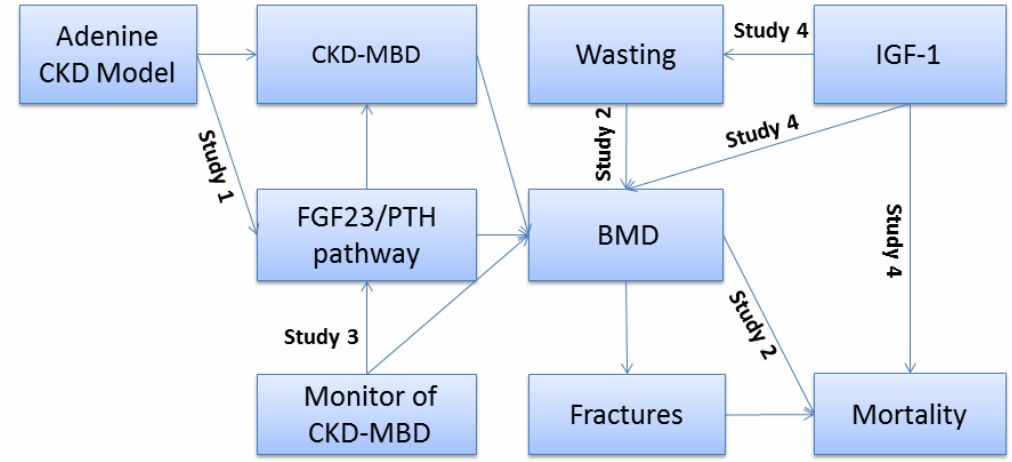

Figure 2.1: Flow chart of the four studies in the current thesis showing the focus of the different studies and how they are linked.

Chapter 3

MATERIALS AND METHODS

3.1 ETHICAL APPROVALS

All studies in this thesis adhere to the Declaration of Helsinki and/or the 3Rs principle (replacement, reduction, refinement), for human and animal studies respectively. **Study 1** followed the guiding principle of animal experiments of Karolinska Institutet (ethical approval numbers: Stockholm South ethical committee S184-10 and Appendix S19-13). **Study 2** and **Study 4** are based on the so called MIA cohort obtained ethical approval from the ethics committee of the Karolinska Institutet (Dnr 273/94; 008/98; 415/03; and 2010/1112). In **Study 3**: studies on the Aachen HD cohort got the ethical permit from the local ethical committee (EK002/04) in Aachen; sutides on the Linköping HDF cohort received ethical approval from the regional ethical committee in Linköping (Dnr M153-07); and, studies on the MIMICK-2 cohort of PD patients received ethical approval from the ethics committee of the Karolinska Institutet (2007/1663-31/4). In studies **2**, **3** and **4**, all participants gave their informed consent.

3.2 PARTICIPANTS

3.2.1 Animal

The housing for the investigated 8-week-old C57BL/6J mice was provided in standard cages with wooden chip bedding and an enrichment of paper rolling and animal were kept at constant ambient temperature (21-22°C) and humidity (45-50%) with 12 hours day-light circle. All animals had free access to tap water and the assigned diet. Before study start, all mice underwent acclimatization to the animal facility conditions and

the casein-based chow for 7 days.

3.2.2 Clinical cohorts

The clinical data in current thesis came from four cohorts: the Malnutrition, Inflammation, and Atherosclerosis (MIA); the Mapping of Inflammation Markers of Chronic Kidney Disease, part 2 (MIMICK-2); the Aachen HD patient cohort and the Linköping HDF cohorts.

3.2.2.1 *MIA*

MIA is a cohort coordinated by the Division of Renal Medicine, Department of Clinical Science, Intervention and Technology, Karolinska Institutet. Patients' phenotype was analyzed post hoc using collected data and, when necessary, by making new analyses from frozen samples. The ongoing MIA cohort study is described in detail elsewhere [109]. Briefly from 1994 to 2009, 434 ESRD patients with GFR <15 mL/min/1.73m^2 were enrolled at Karolinska University Huddinge Hospital at a time-point close to the planned start of dialysis therapy, and were then subject to prospective follow-up with re-investigation in some of the patients after about one year on dialysis treatment. The study exclusion criteria were: age younger than 18 years or older than 70 years, clinical signs of acute infection, active vasculitis or liver disease at the time of evaluation, or unwillingness to participate in the study.

3.2.2.2 *MIMICK-2*

The MIMICK-2 cohort comprises 84 patients from a cross-sectional study with follow-up that originally aimed at monitoring inflammatory markers in all prevalent PD patients who were being controlled at the Karolinska University Hospital and Danderyds Hospital in Stockholm. All participants were prevalent PD patients who had been on continuous ambulatory peritoneal dialysis (CAPD) or automated peritoneal dialysis (APD) for at least 3 months. Patients were recruited from March 2008 to April 2011.

3.2.2.3 *Aachen HD cohort*

The Aachen HD patient cohort involved 56 chronic HD patients who participated in an open-label prospective study. The main target in this cohort was to compare CKD-MBD effects in patients of calcium-containing versus calcium-free phosphate binder regimes. The current post hoc analysis extracted data as previously published [110].

3.2.2.4 *HDF cohort*

Thirty-five patients (30 males, 85%; age 70.8±12.5 years) on chronic HDF were included in the study at the Department of Nephrology, Linköping University Hospital, Linköping, Sweden. Patients were recruited from March 2008 to April 2009. The exclusion criteria were dysfunctional blood access; palliative care (i.e., with reduced dialysis time and death likely to occur within a few weeks) and the inability to speak and understand the Swedish language.

3.3 STUDY PROTOCOLS

3.3.1 Study 1

In **Study 1**, the investigated mice were provided an adenine-containing chow that was prepared by mixing a casein-based diet with different amounts of adenine. Presumable casein could at least in part blunt the smell and taste of adenine. Adenine was purchased by Sigma R&D, and the powdered casein-based diet by Special Diets Services (SDS) (reference number 824522). The diet contained: maize starch (39.31%); casein (20.00%); maltodextrin (14.00%); sucrose (9.23%); maize/corn oil (5%); cellulose (5%); dicalcium phosphate dehydrate (2.36%); disodium hydrogen phosphate anhydrous (1.85%); mineral mix – vitamin E; calcium and phosphate (1.75%); vitamin mix (1.00%); DL-methionine (0.30%) and choline bitartrate (0.20%). Data presented in this paper is derived from an 8-week experiment with 8-week-old C57BL/6 wild-type mice fed either a pure casein diet (n=5; 2 females and 3 males) or a casein-diet supplemented with adenine (n=9; 4 females and 5 males). The protocol of the study is as shown in **Figure 3.1**

3.3.2 Study 2

This is a prospective observational study using data from the MIA-1 cohort. The study included 361 stage 5 patients (218 males, 60%) with a median age of 55 (interquartile range 44-64) years and additionally longitudinal follow-up data were used for survival analysis. No patients were lost to follow-up.

3.3.3 Study 3

This is a prospective observational study using data from the MIMICK-2, Aachen HD and Linköping HDF cohorts. The determinants of FGF23 in patients undergoing PD

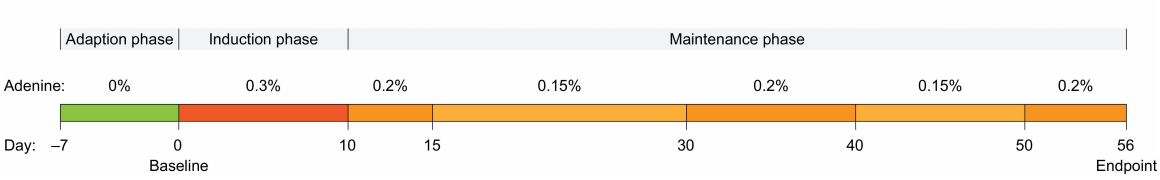

Figure 3.1: The study consisted of a 7-day adaptation phase during which the casein diet was given without the addition of adenine, a 10-day induction phase (day0-9) and a maintenance phase lasting 46 days (day10-56). The adenine doses were modified as described in the protocol of the animal study in the interval 0.15-0.20% to achieve the desired urea level of 80-100 mg/dL

therapy was evaluated in the MIMICK-2 cohort, and the variation of FGF23 in patients receiving different dialysis therapies (PD, HD or HDF) were compared among three cohorts. All PD patients were categorized into a low, median or high variation group based on their tertile of intra-individual coefficient of variation (CVi) of PTH and FGF23. Using receiver operator characteristics (ROC) analysis, the areas under the curve (AUC) of variables associated with PTH and FGF23 variation were calculated. The independent determinants of PTH and FGF23 variation were analyzed with multivariate regression models. The within-subject variation of PTH and FGF23 in different modes of dialysis (PD, HD, HDF) was compared by calculating the intra-class correlation (ICC) from estimates of between-subject σ_b^2 and within-subject variance σ_w^2, derived from two-way mixed effects models, using the following formula**(3.1)**:

$$ICC = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_w^2} \tag{3.1}$$

In the MIMICK-2 cohort, patients (n=6) who had less than three separate timepoints of PTH measurements were excluded. Thus, 78 patients (25 women, 32%; age 63±13 years) were included in the present study. The CKD etiology was chronic glomerulonephritis (14%), diabetic nephropathy (13%), polycystic kidney disease (9%), vascular disease/nephrosclerosis (12%), and miscellaneous/unknown cause (52%). Medication used was as follows: angiotensin converting enzyme inhibitors and angiotensin II receptor blockers (43%), beta-blockers (71%), alpha-blockers (7%), calcium-channel blockers (32%), diuretics (87%), statins (49%), vitamin D receptor activators (83%), phosphate binders (70%), calcimimetics (13%) and warfarin (7%).

In the Aachen HD cohort, patients underwent serial laboratory measurements, including serum concentration of intact-PTH and FGF23, during the study period. Treatment phases were characterized by changes in the type of phosphate binder being used switching between sevelamer and calcium-containing phosphate binders. During the prospective trial, choice of phosphate binders did not have significant impact upon FGF23 concentrations as assessed by repeated measures analysis of covariance (ANCOVA) model. The patients who had less than three measurements of FGF23 or PTH were excluded (n=7). Thus, 49 patients (34 male, 69%; age 65±13 years) were included in the analyses of the present study. The etiology of CKD was diabetic nephropathy (13%); glomerulonephritis (19%); nephrosclerosis (12%); autosomal dominant polycystic kidney disease (14%) and other reasons (21%). The medication used was: angiotensin converting enzyme inhibitors or angiotensin-2 blockers (74%); statins (65%); active vitamin D (79%); cholecalciferol (79%) and phosphate binders (100%). The median HD vintage was 3.1±2.6 years.

In the Linköping HDF cohort, among all 35 patients (30 males, 85%; age 70.8±12.5 years), 31 patients completed 6-months follow-up, and 24 patients 12-months follow-up of online HDF treatment. The reasons for dropping out were death or renal transplantation. The exclusion criteria were dysfunctional blood access, palliative care (i.e., dialysis time had been reduced and death was likely to occur within a few weeks) and the inability to speak and understand the Swedish language. The etiology of CKD was diabetic nephropathy (31%), chronic glomerulonephritis (23%), polycystic kidney disease (17%), vascular disease/nephrosclerosis (14%), interstitial nephritis (3%), drug related nephropathies (3%), congenital malformations (3%), hypertension (3%) and unknown cause (6%). Medication used was as follows: angiotensin converting enzyme inhibitors and angiotensin II receptor blockers (10%), beta-blockers (32%), alpha-blockers (6%), calcium-channel blockers (8%), diuretics (0%), statins (0%), vitamin D receptor activators (88%), phosphate binders (51%), calcimimetics (14%) and warfarin (17%).

3.3.4 Study 4

This is a prospective observational study using data from the MIA cohort. We enrolled 365 CKD stage 5 patients (61% males; median age of 55 years) with IGF-1 measurement and other clinical measurements. The patients attended a second assessment after 1 year of dialysis therapy. Reasons for not attending the second assessment included

death (n=24), kidney transplantation (n=40) and unwillingness or inability to participate (n=57). From the remaining 244 patients, we excluded 10 patients with dialysis duration (vintage) <3 months and 27 additional patients without sufficient serum for analysis of IGF-1 at baseline or 12 months, or both. In the remaining subgroup of 207 patients (62% males; median age of 55 years), a follow-up investigation was performed. Most patients had antihypertensive medications (98%); phosphate binders (81%) and diuretics (82%). Vitamin B, C, and D (73% received oral vitamin D analogues) were supplemented in accordance with clinical practice. We analyzed the baseline serum IGF-1 concentration and the longitudinal change of IGF-1 over one year in CKD stage 5 patients starting on dialysis in relation to BMD, nutritional status, metabolic parameters and mortality. We compared patients with 1) high IGF-1 both at baseline and one year, or shifted from low to high IGF-1, *persistently high/increasing group* with 2) patients with low IGF-1, both at baseline and at one year, or declined from high to low IGF1, *persistently low/decreasing group.*

3.4 METHODS

3.4.1 Clinical examination

In the MIA cohort, glomerular filtration rate (GFR) was estimated as the mean of urea and creatinine clearance from 24-hour urinary samples. Dual energy X-ray absorptiometry (DEXA) was performed using the DPX-L device (Lunar Corp., Madison, WI, USA) to measure total BMD, total fat mass and lean body mass. The distribution was directly estimated without making assumptions about the two-compartment model. In addition, seven areal BMD (g/cm^2) were simultaneously measured with DEXA, including head, arms, legs, trunk, hip, pelvis and spine. Total BMD was expressed as a T-score, indicating the number of standard deviations (SD) from the mean scores for 30-year old normal men and women separately. The Z-score indicates the T-score adjusted by age. The same instrument and methods were used for the entire study period. Osteoporosis was defined by a T-score < -2.5, osteopenia: -2.5 <T-score < -1, and normal BMD: T-score>-1.

Hand grip strength was evaluated using a Harpenden dynamometer bilaterally, using data from the dominant arm (usually right arm) as many patients had an arteriovenous fistula in the non-dominant arm. Nutritional status was assessed by means of subjective global assessment (SGA) at the time of inclusion, concurrent with drawing of blood

samples. Body mass index was calculated as following formula **(3.2)**

$$BMI = \frac{Body\ Weight(kg)}{Height(m^2)} \tag{3.2}$$

3.4.2 Laboratory analyses

In the animal study (**Study 1**), serum was collected by tail vein incision at intermediary time points during the study and during cardiac puncture when the animals were sacrificed. A urine sample was collected after spontaneous urination. Serum and urine biomarkers, such as calcium, phosphate, creatinine and urea, were measured with Konelab 20XTi (Thermo Scientific, Finland) research platform. Creatinine concentrations were validated with a colorimetric assay (BioChain, CA, USA). The results were nearly identical (rho=0.95 and 0.98 for serum and urine creatinine respectively). PTH was measured by commercial ELISA kit for measuring intact PTH (Immutopics, CA, USA), FGF23 with an intact FGF23 ELISA (Kainos, Japan) and 1,25(OH)2D, CTX and PINP with EIA kits (Immunodiagnostic Systems, United Kingdom).

In the MIA cohort, venous blood samples were drawn after an overnight fast, and stored at -70° C for biochemical analyses. Serum osteoprotegerin (OPG) was analyzed by ELISA using a commercially available kit (R&D Systems Inc, Minneapolis, MN, USA). Leptin concentration in serum was measured by a radioimmunoassay kit (Linco Research Inc, St. Charles, MO, USA) and adiponectin by a commercially available ELISA kit (Linco Research Inc.). ELISA plates were read using a Spectra MAX (Molecular Devices Corp, Sunnyvale, CA) instrument, and data were analyzed using the SoftmaxPRO software (Molecular Devices Corp.). Plasma levels of IL-6, IGF-I, and IGF-binding protein 1 and 3 (IGFBP-1 and IGFBP-3) were measured using immunometric assays on an Immulite Automatic Analyzer on an Immulite Analyzer (DPC Corp., Los Angeles, CA). The intra-assay coefficient of variation (CV) for IGF-1 was 4.3%, and the inter-assay CV was 6.9%.

Soluble intercellular adhesion molecule (s-ICAM)-1 and soluble vascular cell adhesion molecule (VCAM)-1 was measured using commercially available assays (R&D Systems Europe Ltd, Abingdon, UK). FGF23 was measured using an ELISA recogniz-

ing the intact, biologically active FGF23 (Kainos Laboratories International, Tokyo, Japan). The remaining biochemical analyses (including intact PTH, phosphate, calcium, albumin, creatinine, hemoglobin) were performed using routine methods at the Department of Clinical Chemistry at Karolinska University Hospital, Huddinge. The adjusted calcium levels were calculated using Payne's formula [111].

3.4.3 Histology

Kidneys and parathyroid glands were collected and then fixed into 4% formaldehyde after harvest. The tissues were than embedded in paraffin. The sectioned progress was done according to standard procedures. Bones were decalcified in a buffer with 20% formic acid. Hematoxylin and Eosin staining were done in all tissues. Kidneys and bones from adenine-treated mice and controls were evaluated in a blinded fashion by experienced kidney pathologist (A.W.) and bone pathologist (G.A.). Rabbit monoclonal anti-Ki67 antibody (SP6 1:400, Thermo Scientific, Fremont, CA, US) was used for immunohistochemistry analysis according to standard protocols. The proliferation index in parathyroid glands was calculated by the number of Ki67 positive cells divided by the total number of cells of four consecutive sections.

3.4.4 Follow-up

The patients in the MIA cohort were prospectively followed-up for up to 5 years, or until April 30^{th}, 2011, or until events of death or kidney transplantation occurred, whatever came first. Causes of death were extracted from medical recorded by a physician blind to the study results.

3.4.5 Statistical analysis

Values were expressed as mean ± standard deviation, median (interquartile range; IQR) or as percentage of total, as appropriate. Logarithmic transformation was applied for non-normally distributed continuous valuables. All tests were two-tailed and $P<0.05$ was considered significant. All statistical analyses were performed using statistical software STATA version 12 (Stata Corporation, College Station, TX, USA) and GraphPad Prism version 5.0 or higher (GraphPad Software Inc, CA, USA).

Comparisons of continuous variables among groups were performed using ANOVA or Kruskal-Wallis tests. Comparison of nominal variables among these groups was

performed using χ^2 test. Spearman's rank correlation (ρ) was used to determine correlations of variables.

Survival analyses were made with the Kaplan-Meier survival curve and the Cox proportional hazard model. Restricted cubic splines were used to evaluate the non-linear relationships between the studied variable and outcome. The univariate and multivariate Cox regression analysis are presented as hazard ratio (HR; 95% confidence intervals (CI)). Because the kidney transplantation rate of patients in the MIA cohort was high, to avoid bias in traditional Cox regression models, we analyzed the cumulative incidence of death before kidney transplantation, and the applied the competing risk approach. Data are presented as hazard ratios and 95% confidence intervals.

Other particular statistical analyses are discussed in each of the studies presented in this thesis.

Chapter 4

MAIN RESULTS AND DISCUSSIONS

4.1 STRENGTHS AND LIMITATIONS

4.1.1 Strengths

The studies presented in this thesis contribute to the knowledge of mineral and bone disorders and their clinical implications in ESRD patients. In addition, we expand the knowledge regarding methods to experimentally induced changes in the phenotype of mineral and bone disorders and associated biomarkers using a non-surgical renal failure model in mice. Thus, in **Study 1**, we showed that using adenine such a model is feasible and could be a suitable method for inducing CKD-MBD phenotype changes without the need for complicated technology and potentially traumatic surgical procedures in mice. Among the three clinical studies, **Study 2** provided detailed information of determinants of low BMD in ESRD patients, and assessed the implications of the abnormal bone status, especially osteoporosis, for the all-cause mortality risk in these patients. **Study 3** was an exploratory study comparing the monitoring performance of the relatively novel CKD-MBD marker FGF23 with a traditional marker hitherto recommended by guidelines, namely PTH. The analyses of determinants of the variability of these two markers gave information of potential relevance for the clinical application of these two CKD-MBD markers. Our data emphasize for the first time that - because of its lower variability - FGF23 may be preferred as a reliable biomarker of CKD-MBD in dialysis patients. Finally, **Study 4** filled the gap of previously not existing knowledge of the important relation between circulating IGF-1, bone status and mortality in

ESRD patients, exploring correlates of IGF-1 at different time points, and its changes during one year of dialysis, in relation to mortality risk in incident dialysis patients.

4.1.2 Limitations

The results in this thesis should be interpreted with some caution considering the study limitations. In the animal study (**Study1**), the underlying etiology of adenine-induced nephropathy is tubular toxicity, which differs from human CKD in that the most common pathology in CKD patients is glomerular scarring. This model should be regarded however not as a model of tubule-interstitial disease with declined renal function but rather as a model of CKD-BMD phenotype. It should be noted that adenine may have side-effects besides its impact on the kidneys, and these side-effects could potentially influence the phenotype independently of uremia. Furthermore, the reversibility of renal failure, or long-term impact of adenine administration has not been studied yet.

In the clinical studies (**Study 2, 3,** and **4**), the cross-sectional nature of analyses does not allow inferring causality from the results. Also, there could be residual confounding due to many unmeasured or unknown confounding factors that we cannot take into account. Thirdly, due to the selection of patients, the study cohorts may not be representative of unselected patients. For example, in **Study 2**, mortality rate and especially incidence of fractures were lower than those reported in other European ESRD populations [112; 113]. This could be due at least in part to our patients being clinically more stable and on average younger, as compared to unselected patient cohorts and also due to the fact that the MIA study was not focused on the study of incidence of fractures. In addition, this and the other clinical studies in this thesis suffer from lack of details regarding bone status which could have been provided by methods such as bone biopsy. In **Study 3**, the assay for measurement of FGF23 was different in the PD/HDF cohorts (intact assay) and the HD cohort (C-terminal assay, detecting both intact and any C-terminal FGF23 fragments). Seasonal variation in vitamin D status and dietary differences in vitamin D, calcium and phosphorous have not been considered, and urinary phosphorous and calcium excretion were not monitored in patients with residual urinary output. In **Study 4**, only those patients who survived and were willing to participate in the follow-up investigation at one year participated in the longitudinal study; this may lead to biased conclusions regarding links between IGF-1 changes and survival. Furthermore, we only analyzed a restricted amount of variables; again it should be noted that inclusion of unmeasured potential confounding factors

such as growth hormone, IGFBP-1, IGFBP-3 (other than at baseline), IGFBP-4, and indices of glucose metabolism and genetic factors could have changed the conclusions.

4.2 RESULTS and GENERAL DISCUSSION

4.2.1 Study 1

In the animal study (**Study 1**), we established a CKD model which could be used to study the bone mineral disorders in an animal model in mice. The significant decline of body weight during the induction period have some similarity with the development of sarcopenia and wasting in CKD patients [114]. **Figure 4.1** shows the temporal pattern of selected markers of bone and mineral metabolism. The adenine group developed hyperphosphatemia, secondary hyperparathyroidism and high FGF23, in parallel with an increased urinary excretion of phosphorous; these changes are similar to those observed in CKD patients. The level of CTX decreased significantly and that of PINP increased with borderline significance in the adenine-treated group at the end point, suggesting increased bone formation.

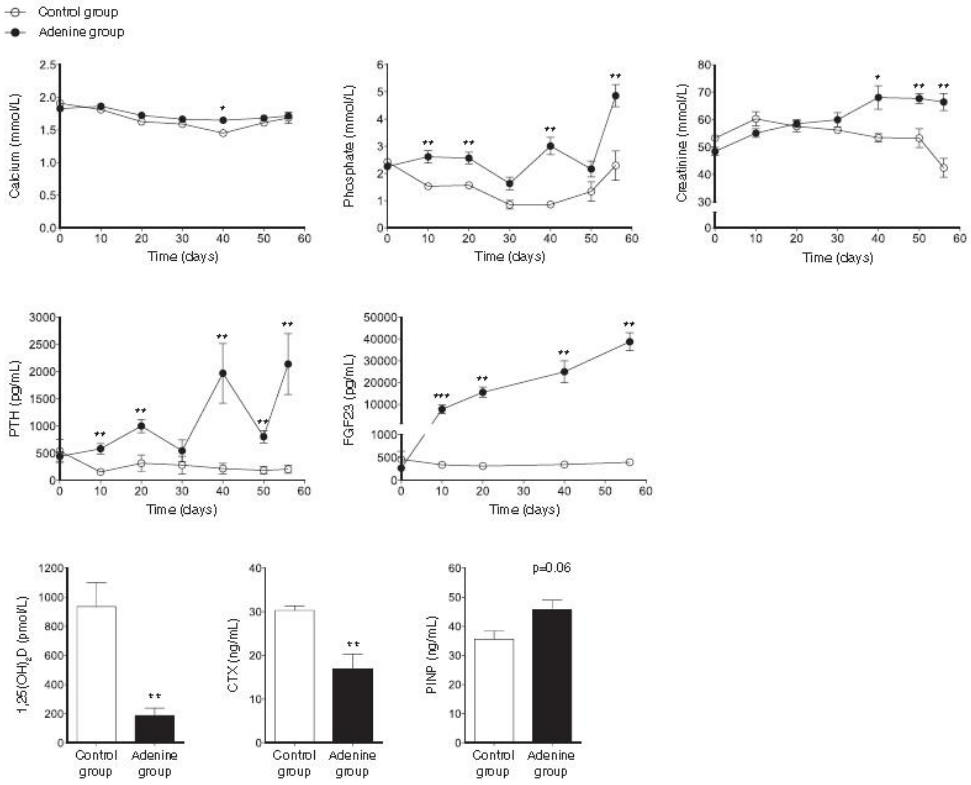

Figure 4.1: Temporal changes in serum biochemistries of mineral metabolism

There are several biochemical findings that deserve mentioning. The serum calcium level was stable in the adenine-treated mice likely due to a compensatory rise in PTH, with a reduction of 1,25(OH)2D, which largely mirrors the situation in CKD patients stages 3-4 [115]. Normal serum calcium concentrations have also been reported in other CKD models [116]. Another interesting finding is that the FGF23 concentration increases continuously, whereas other bone-mineral markers remained constant during the maintenance phase of the study. This suggests that renal-specific factors, and not only renal function *per se*, stimulates FGF23 synthesis in bone. The bone markers conceivably reflect an increase in bone formation and a decrease in resorption, which may be unexpected in a uremic model with secondary hyperparathyroidism. The mechanisms behind the finding of reduced bone resorption are unclear; possibly this change could be due to impaired osteoclast function due to physiological suppression from the elevated FGF23 [117].

The expression of renal-derived inflammatory and fibrosis genes increased in the adenine group as shown in Figure **4.2 A** and **B**. The expression of inflammation genes was up-regulated, which mirrors the inflammation status in CKD patients [118]. Bone sections from the femur showed extended bone trabecular and increased bone marrow adiposity. This finding is consistent with the changed pattern of the bone markers PINP and CTX as shown in **Figure 4.3**.

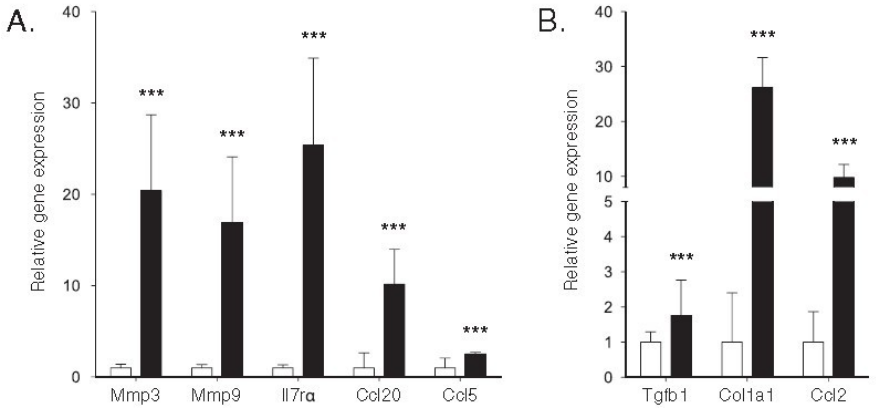

Figure 4.2: Expression of renal-derived inflammatory and fibrosis genes. **A)** Inflammatory markers Mmp3, Mmp9, Il7ra, Ccl20 and Ccl5, and **B)** markers of fibrosis Tgfb1, Col1a1 and Ccl2 were upregulated in the adenine group. Gene expression was expressed in relation to that in the control group of respective control gene (Set as 1). White bars, control group; black bars; CKD group induced by adenine.

Renal histology showed mainly tubule-interstitial damage with peritubular leukocyte infiltration and interstitial/peritubular edema. Some of the adenine-exposed mice exhibited signs of changes in the glomeruli. The von Kossa stain revealed extensive calcification of tubular structures. The proliferation ratio of parathyroid glands also increased.

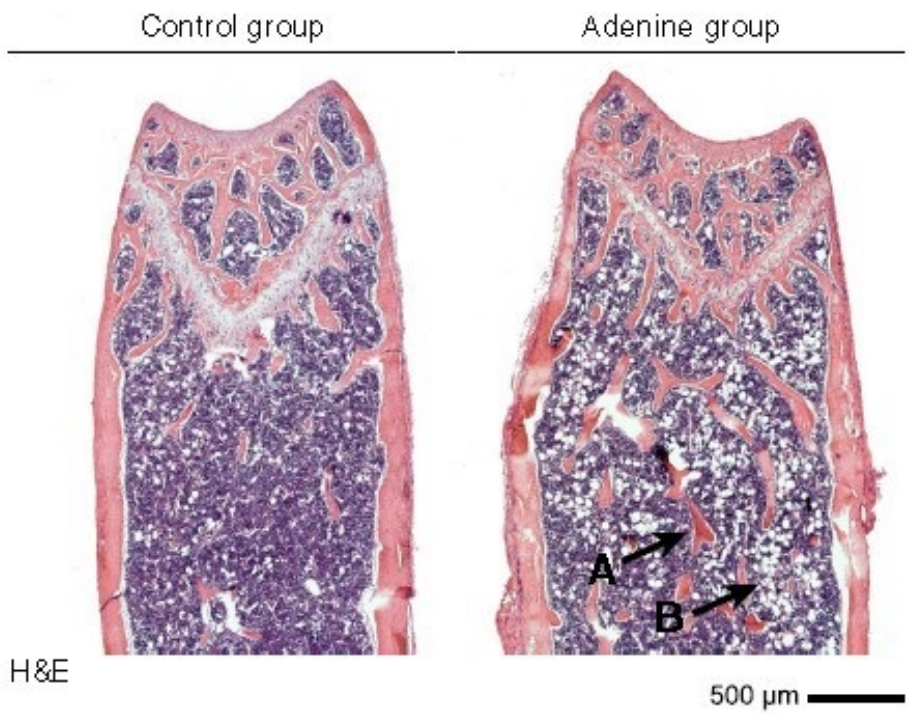

Figure 4.3: The number and thickness of submetaphyseal bone trabeculae increased in the bone (arrow A); and, the adipocyte content increased in the bone marrow (B)

Established techniques for induction of renal failure in mice are mostly dependent on surgical interventions. The most widely used methods currently are unilateral ureteral obstruction [119; 120] and 5/6 nephrectomy [121; 122]. In **Study 1**, we developed a novel adenine-based protocol to induce tubule-interstitial nephropathy in mice. The model showed several features of human CKD-MBD, such as changes in bone turnover, loss of body weight, and wasting and increased expression of genes related to inflammation and fibrosis. This less complicated non-surgical animal model could be used to explore the mechanism of CKD-BMD in mice. Another benefit of this model is the adjustable level of uremia which could be modified by altering the adenine dose. Additional advantages of this model in our hands include zero mortality. This model therefore allows using a limited number of animals to establish a CKD model whereas surgical methods may have a higher mortality. Also, the model applied presented a smaller inter-individual variation in renal function compared with that reported using the 5/6 nephrectomy model [123; 124]. There are also some other non-surgical ure-

mic mice models, including nephropathy induced by radiation and administration of nephrotoxic drugs, such as folic acid [125], cyclosporine A [126] and cisplatin [127]; however, these techniques may result in non-reversible kidney damage.

In this protocol there is no need of surgical procedures making it practical for those with no rodent operation skills. We should emphasize that genetically modified strains so far mainly exist in mice. The model in the current study may serve as an important complement to existing study models for exploring the gene function in the setting of impaired renal function.

4.2.2 Study 2

BMD is a widely used component in the assessment of osteoporosis and the risk of fractures, as well as for monitoring the natural history of treated and untreated bone disease. In **Study 2**, we studied determinants of BMD and the implications of low BMD in terms of increased mortality. The median T-score was -0.6 (range, -4.8 to 3.0), indicating that bone mass was slightly lower compared with the bone mass in sex-matched healthy subjects, and 37% of the subjects had signs of osteopenia/osteoporosis. Univariate correlations between T-score and several parameters, represented by Spearman's rank correlation coefficient, are presented in **Table 4.1**.

Table 4.1: Correlations between T-score and clinical and laboratory parameters in ESRD patients

Variable	*Rho (Spearman)*	P value
Age, years (n=361)	-0.22	<0.001
Body weight, kg (n=361)	0.389	<0.001
BMI, kg/m^2 **(n=361)**	0.375	<0.001
GFR, mL/min (n=276)	-0.001	0.999
Body composition		
***Lean body mass*, kg (n=350)**	0.278	<0.001
***Body fat mass*, kg (n=350)**	0.308	<0.001
Nutritional markers		
Albumin, g/L *(n=361)*	-0.03	0.596
***Handgrip strength* (n=326)**	0.292	<0.001
***SGA* (n=343)**	-0.274	<0.001
***Hb*, g/L (n=356)**	-0.076	0.15
***Creatinine*, μmol/L (n=351)**	0.31	<0.001
Bone-related markers		
Calcium*, mmol/l (n=337)*	0.048	0.383
***Phosphate*, mmol/L (n=333)**	0.174	0.001
***PTH*, ng/L (n=353)**	-0.088	0.098
***OPG*, pg/mL (n=222)**	-0.164	0.014
Inflammatory markers		
***hs-CRP*, mg/L (n=353)**	-0.057	0.284
***IL-6*, pg/mL (n=326)**	-0.118	0.033
***VCAM-1*, ng/mL (n=275)**	-0.058	0.335
***ICAM-1*, ng/mL (n=275)**	-0.21	<0.001
Adipokines		
***S-leptin*, ng/mL (n=313)**	0.124	0.028
IGF axis		
***IGF-I*, ng/mL (n=204)**	0.301	<0.001
***IGF-BP1*, ng/mL (n=204)**	-0.342	<0.001
***IGF-BP3*, ng/mL (n=207)**	0.231	<0.001

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; SGA, subjective global assessment; iPTH, intact parathyroid hormone; hs-CRP, high sensitivity C-reactive protein; IL-6, interleukin-6; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IGF-1, insulin-like growth factor; IGFBP, IGF binding protein. The numbers in parenthesis mean the number of patients included in each analysis

The multivariate regression model indicated that, age, total fat mass, wasting and PTH were predictors of T-score at the time of the baseline investigation. More impor-

tantly, with 5 years follow-up, we found that patients with osteopenia/osteoporosis had a higher mortality risk (HR=2.4, 95% CI, 1.3-4.6) than the patients with normal bone mass. In multivariate Cox proportional hazards analysis, T-score higher than -1 was related with 17.6% lower mortality risk (Hazard ratio, 0.824, 95% CI, 0.681-0.996) after adjustment for risk factors of low BMD, such as age, fat mass and malnutrition/wasting (**Figure 4.4**).

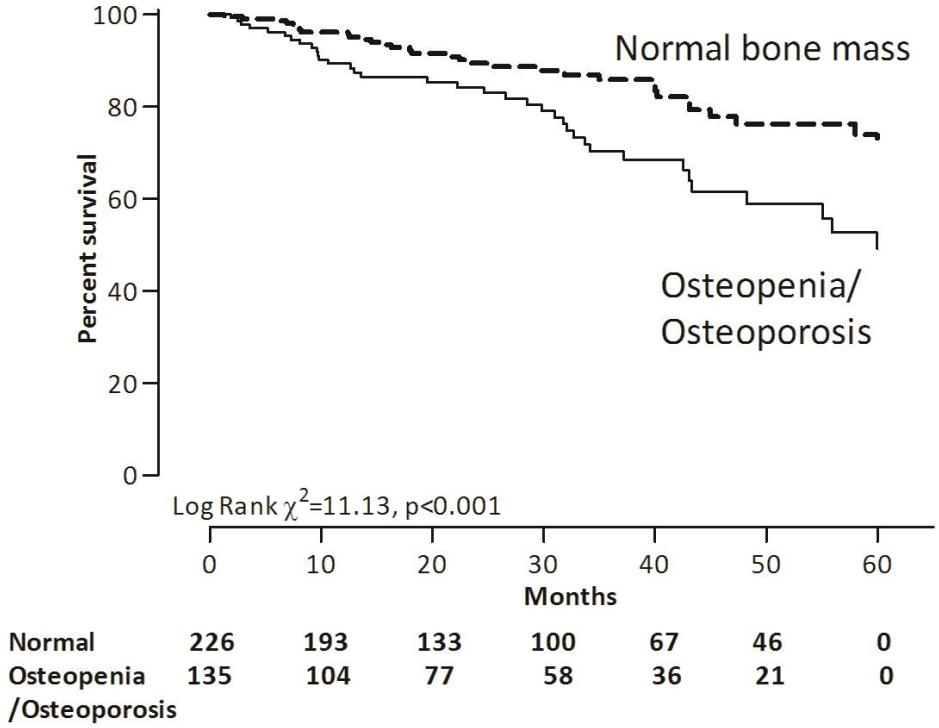

Figure 4.4: Kaplan-Meier survival curve for all-cause mortality over 5 years of follow-up in ESRD patients with normal BMD (T-score>-1) and with osteopenia/osteoporosis (T-score <-1) respectively

The positive correlation between body weight and BMD is thought to be mainly due to an impact on bone remodeling through load stress [19]. The results in **Study 2** confirmed this link between body weight and BMD suggesting that load stress promotes bone formation also in the setting of progressive renal failure. The results in **Study 2** also suggested that the IGF-1 system (IGF-1 as such as well as the IGF-1 binding proteins, IGF-BP1 and IGF-BP3) are important for BMD in ESRD patients.

This has been described previously in the general population [128]; however, our study is the first one that documented such an association in ESRD patients. Considering that IGF-1 is an essential regulator of bone growth with low values reflecting early changes of bone mass in pre- and post-menopausal women [64], IGF-1 could represent another biomarker for monitoring bone status; this is discussed in more detail below, see **Study 4**.

FGF23 is a bone-derived circulating factor that decreases serum concentrations of inorganic phosphorous (Pi) and 1, 25-dihydroxyvitamin D [129]. FGF23 associates with fracture risk in old men [130] and post-transplant loss of bone mass [131]. In the current study, the FGF23 level in the normal bone mass group was slightly higher than in the bone loss group (p=0.05), but there was no association between fracture risk and FGF23, possibly because of the limited number of fractures (n=9).

One possible reason for the association between high mortality and low BMD could be that CKD patients commonly suffer from adynamic bone disease [132]. In that situation, bone is incapable of buffering calcium and phosphate, leading to more extensive vascular calcification and cardiovascular disease [133]. We should emphasize that the definition of osteoporosis and the appropriate diagnostic tools for detecting osteoporosis in CKD patients are controversial [134]. To determine if adynamic bone disease is present or not, considering that bone biopsies are seldom clinically feasible, measurements of alkaline phosphatase and other bone resorption markers, such as serum osteocalcin, tartrate-resistance acid phosphatase 5b (TRAP5b) or bone-specific alkaline phosphatase have been proposed. The accuracy, both sensitivity and specificity, of such measurements are however not clear; further studies are needed in this area.

4.2.3 Study 3

Hyperphosphatemia and secondary hyperparathyroidism are two mineral metabolism abnormalities in patients with CKD, which are especially important in ESRD patients receiving dialysis treatment, as they are closely related with high mortality [135]. Most of the therapeutic strategies for the treatment of bone mineral disorders in CKD are based on monitoring surrogate biomarkers such as calcium, phosphorus and PTH regularly, to assess whether values are within target ranges defined by clinical guidelines [136]. Monitoring PTH is a cornerstone in the clinical care of patients with CKD-MBD. In the clinical setting, therapeutic modifications of medications targeting CKD-MBD,

such as phosphate binders, vitamin D analogues and calcimimetics, are often based on changes in the circulating PTH concentration [137]. In addition to PTH, FGF23 is a novel CKD-MBD biomarker, which changes early in the course of CKD progression, preceding changes in phosphate and PTH, and associates with adverse cardiovascular outcomes [138]. Thus, routine clinical measurement of FGF23 may enable earlier identification of individuals with CKD-MBD [139]. On the other hand, PTH is not an ideal biomarker of CKD-MBD considering its large temporal intra-individual variation, implying that treatment choices based on PTH could be mainly based on the large biological variability of PTH rather than on underlying pathophysiological processes [140]. In order to validate the use of PTH as a clinical biomarker for monitoring CKD-MBD, we need more studies about determinants of its high variability in the clinical setting.

In **Study 3**, we evaluated determinants of the intra-subject variability of two CKD-MBD markers, PTH and FGF23, in a cohort of PD patients and then analyzed PTH and FGF23 variability in patients receiving different dialysis treatments, such as PD, HD and online HDF (**Table4.2**).

Table 4.2: Predictors of FGF23 and PTH variability, expressed as Spearman's correlation coefficient, and as area under the curve (AUC) from receiver operator characteristics (ROC) curves for selected variables, representing various profiles, that significantly differed in their intra-individual coefficient of variation for lgFGF23 and PTH at baseline among the PD patients.

Dependents	**Profiles**	**Variables**	**beta**	**AUC**	**SE**
lgFGF23 variability	**General**	Gender (M/F)	-0.23^a	0.64	1.28
	MBD	Phosphate (mg/dL)	-0.29^a	**0.72**^{a}	1.13
	Lipid	HDL (mg/dL)	0.19^a	0.63	-0.53
	Thyroid	TSH (mIU/L)	0.21^a	0.65	-0.14
PTH variability	**General**	Age (years)	-0.20^a	0.61	0.03
	MBD	25(OH)D (ng/mL)	-0.20a	**0.75**^{a}	0.06
	Nutrition	PEW % (SGA>1)	0.35^b	**0.70**^{a}	-1.18
		Fat mass (kg)	-0.36^b	**0.71**^{a}	0.07
	Diabetes	Glucose (mg/dL)	-0.21^a	0.63	0.07
		Insulin (μU/mL)	0.20^a	0.63	0.003
	Lipid	Triglycerides (mg/dL)	-0.20^a	0.67	0.53

Note: All models were adjusted for age, gender and Davies scores. MBD, mineral bone disorder; HDL, high density lipoprotein; TSH, thyroid-stimulating hormone; AUC, area under curve; PEW, protein-energy wasting; ^{a}p<0.05; ^{b}p<0.01 CVi was defined as the coefficient of variation and calculated as the ratio between the standard deviation and mean

In a multivariate stepwise regression model (**Table 4.3**) including significant parameters from Spearman's correlations, presence of PEW and lower fat mass predicted high PTH variability, whereas associations with lower triglycerides and 25(OH)D concentrations were borderline significant. Interestingly, and not unexpected, lower phosphorous was the only predictive factor of high FGF23 variability. **Figure 4.5** represents the ICC estimates of lg PTH and lgFGF23 according to dialysis modes. Importantly, lgFGF23 had the highest ICC estimate (lowest intra-individual variation) in all groups of patients.).

Table 4.3: Stepwise regression model showing significant predictors of variability for lgFGF23 and lgPTH in PD patients

		lgFGF23 Variation		**PTH Variation**	
Profiles	**Variables**	Standard beta-value	*p*	Standard beta-value	*p*
MBD	Phosphorous, mmol/L	-0.23	0.03		
	25(OH)D, ng/mL			-0.2	0.08
Nutrition	PEW (SGA>1),%			0.3	0.04
	Fat mass, kg			-0.38	<0.001
Lipid	Triglycerides, mmol/L			-0.22	0.06

Note: Variables included in the model were selected on the basis of a univariate correlation with either lgFGF23 or PTH variability at the significance level of p<0.10. MBD, mineral bone disorder; PEW, protein energy wasting.

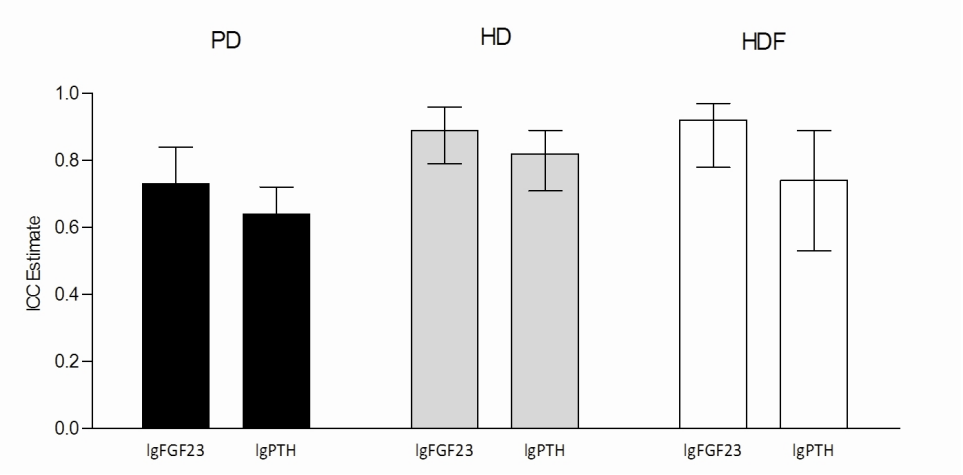

Figure 4.5: Intra-class correlation for lgFGF23 and lgPTH in different modes of dialysis (PD, HD and HDF)

Only a few studies have explored the longitudinal variation of mineral metabolism markers in PD patients and none of these included analyses of determinants of variability. Numerous reports have described the large PTH variations in CKD patients as well as in the general population [140; 141; 142]. One factor of importance is that PTH measurements have high inter-method variability since commercially available assays have different antibody specificity and cross-reactivity for the various PTH fragments [143]. We reported, for the first time, that the intra-individual PTH variation was larger than that for FGF23, irrespective of dialysis modality. Consistent with previous studies [141; 144], we found that a lower 25(OH)D concentration was related with high PTH variability; this is biologically plausible given that vitamin D deficiency increases the risk for hypocalcemia and parallel dynamic changes in PTH. Importantly, we found that the serum phosphorous concentration had a close relationship with FGF23 variability; this is consistent with previous observational studies showing that circulating phosphorous, in addition to GFR, is the strongest determinant of FGF23 in CKD patients [145].

Interestingly, metabolic features not primarily thought to be related to mineral metabolism were associated with greater PTH variability, including lower fat mass and presence of protein energy wasting (PEW). The assessments of these clinical parameters are relatively inexpensive, easily obtainable and may be useful as simple screening tools to identify individuals whom may benefit from repeated PTH measurements. Individuals with malnutrition/PEW have lower phosphorous concentrations than healthy subjects. Since PTH is a potent phosphaturic hormone, its variability may increase with lower phosphorous concentrations as observed for FGF23. In turn, vitamin D concentrations are intimately related with fat mass and other metabolic derangements as showed in large observational studies [146; 147].

The current study showed that female subjects and patients with higher high-density lipoprotein (HDL) cholesterol and thyroid stimulating hormone (TSH) had greater FGF23 variability in crude analysis. The mechanisms behind the observed positive association between HDL cholesterol and FGF23 variability is unclear, but in individuals with normal kidney function, previous studies demonstrated an inverse correlation between FGF23 and HDL cholesterol concentrations [148] also in ESRD subjects [149], suggesting a biological interaction between this mineral marker and the lipid profile.

Finally, we found in **Study 3** that the intra-individual stability of FGF23 was

greater than that for PTH in PD, HD and HDF patients, favoring FGF23 as a suitable biomarker in terms of stability. These results are consistent with another report comparing PTH and FGF23 variability in PD patients [150] which we now extended to the HD and HDF dialysis populations. It should be noted that, the comparably lower longitudinal FGF23 variability is in sharp contrast to the exceptionally high inter-individual variation observed amongst ESRD subjects [138]. The added value of including FGF23 measurements in dialysis patients is however unclear, in particular since no therapeutic interventions currently are available to lower its concentrations effectively.

4.2.4 Study 4

The IGF-1 system plays an important role for body growth and body composition and it is well established that IGF-1 can block skeletal muscle wasting [151]. IGF-1 is also closely related to bone mineral metabolism and risk of fractures in females as described previously [152]. Many CKD patients display signs of an abnormal of IGF-1 system with decreased circulating levels of IGF-1 and also resistance to the function of IGF-1 [153]. In **Study 2** we could show that IGF-1 is a predictor of BMD in CKD patients. Since a low IGF-1 level independently relates with increased risk of ischemic heart diseases in elderly subjects [154], and a low IGF-1 level in CKD patients links closely to impairments in body composition, particularly muscle wasting and reduced BMD [155], we hypothesized that a low IGF-1 concentration could be linked with increased mortality in CKD patients starting on dialysis. We studied in **Study 4** the longitudinal change of IGF-1 over 1 year of dialysis, and the metabolic links of IGF-1 with bone mineral markers and, nutritional status. The IGF-1 concentration increased after one year dialysis treatment regardless of the dialysis mode, PD or HD. **Table 4.4** reports the univariate associations between IGF-1 at the initiation of dialysis in all patients. The significant association between IGF-1 and bone mineral makers such as OPG, FGF23 and BMD is in line with the mechanistic links between the IGF-1 system and mineral and bone metabolism reported in **Study 2**.

Table 4.4: Univariate associations expressed as standardized β coefficients between IGF-1 and other variables at the initiation of dialysis in all 365 CKD stage 5 patients initiating dialysis

	All patients (n=365)	
Characteristics	β	P value
Age, years	-0.27	<0.001
Male, %	0.03	0.68
DM, present	-0.24	<0.001
CVD, present	-0.23	0.001
SGA >1, %	-0.17	0.02
IL-6, pg/mL	-0.13	0.05
Phosphate, mg/dL	0.16	0.03
Calcium, mg/dL	0.16	0.03
PTH, ng/L	-0.09	0.18
Fat mass, kg	0.14	0.04
Lean body mass, kg	-0.06	0.4
Total BMD, g/cm^2	0.18	0.03
FGF-23, pg/ml	0.27	0.002
OPG, pg/ml	-0.18	0.03

Note: DM, diabetes mellitus; CVD, cardiovascular disease; SGA, subjective global assessment; PTH, parathyroid hormone; BMD, body bone mineral density; FGF23, fibroblast growth factor-23; OPG, osteoprotegerin.

During a median of 5 years follow-up, we found a gradual decrease of HR for all-cause mortality with increased IGF-1 level (**Figure 4.6**). Patients who showed persistently low or decreased IGF-1 concentration during the first year of dialysis had increased mortality compared with patients who had persistently high or increased IGF-1 concentration (**Figure 4.7**). In further analysis considering dialysis treatments, we found a significant association between 1-SD IGF-1with low mortality in HD patients, not in PD patients.

As far as we know, no previous study addressed the mortality risk associated with change in IGF-1 levels occurring in ESRD patients during their first year on dialysis and no previous study analyzed the possible impact of different dialysis treatments.

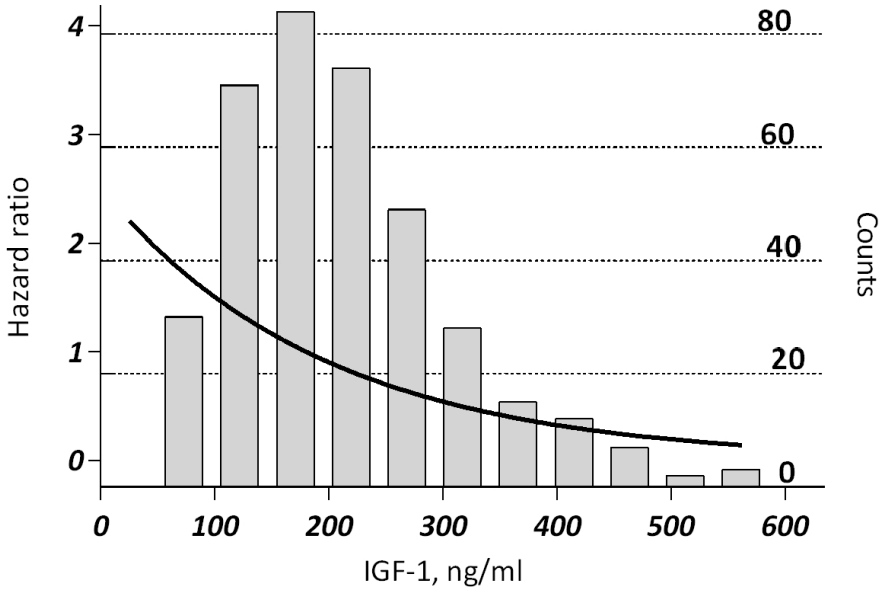

Figure 4.6: Spline curve showing the IGF-1 levels at baseline and the predictive hazard ratio for all-cause mortality of a median of 5 years follow-up in all 365 patients. The bars represent the number of patients for each IGF-1 interval

Among possible reasons why higher IGF-1 associates with better survival are that IGF-1 enhances protein synthesis and nitrogen balance and improves bone growth; but, also that IGF-1 is involved in the regulation of glucose metabolism and may suppress lipolysis [156].

In **Study 4**, we could demonstrate that IGF-1 displayed strong links with mineral and bone metabolism parameters in CKD stage 5 patients. These results confirm previous results in animal studies showing that circulating IGF-1 plays a key role as a regulator of bone growth and BMD [157], and suggest that a low IGF-1 may be a key factor for the mineral and bone disorders in CKD patients. A decrease of IGF-1 could also be a primary cause of decreased BMD occurring with ageing [158]; this is of importance also for CKD considering the advanced age of most CKD patients. Whereas we did not find a significant association between circulating IGF-1 and PTH levels, both calcium and phosphate were identified as predictors of IGF-1 changes occurring during 1 year of dialysis treatment as analyzed by the mixed model applied in **Study 4**; this

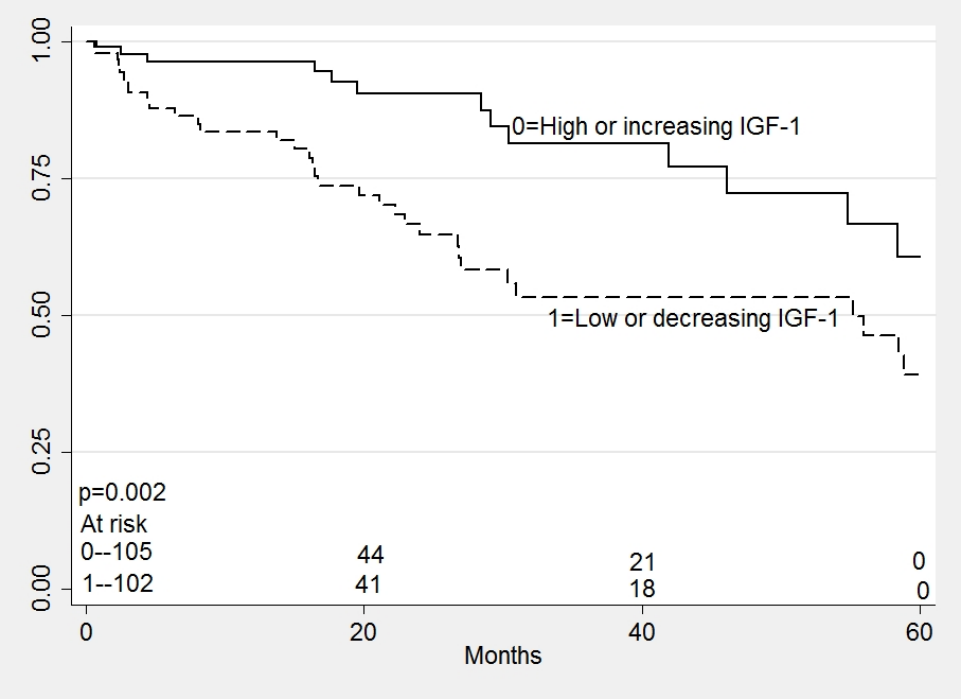

Figure 4.7: Kaplan-Meier curves of survival for a median of 5 years follow-up of 207 patients who had persistently high or increased IGF-1 levels, or persistently low or decreased IGF-1 levels during their first year on dialysis. *Persistently high/increased group* was defined as patients with high IGF-1 both at baseline and at one year, or shifted from low to high IGF-1; *persistently low/decreased group* was defined as patients with low IGF-1, both at baseline and at one year, or declined from high to low IGF-1.

may suggest that the IGF-1 system directly or indirectly can affect phosphate through the PTH pathway although our data could not confirm this. Furthermore, phosphate and calcium modulate the osteoblastic proliferation through IGF-1 in in vivo studies [128; 159]. Also, it has been reported that the effect of PTH on bone-forming requires the involvement of the IGF-1 system, suggesting that IGF-1 plays a role as a potential mediator of the anabolic action of PTH [160]. *Vice versa*, earlier studies revealed that PTH can stimulate IGF-1 production in osteoblasts [161].

Studies of relationships between FGF23 and BMD in previous studies showed conflicting results. One study in patients with CKD stages 2 – 5 found no association between FGF23 and BMD [48], and another study in HD patients found no association as well [16]. In the current study, FGF23 was positively related to IGF-1 at baseline; this finding is in accordance with other studies in ESRD and CKD patients showing a

relation between BMD and FGF23 [69; 162]. These results may be linked to the finding that IGF-1 is involved as an NF-κB ligand in the FGF-23/Klotho pathways [163].

It may seem contradictory that in **Study 4**, IGF-1 was positively related to phosphate and FGF23, two potential mortality risk markers, but inversely related to presence of DM and CVD. However, it is likely that while the observed positive trilateral links between the anabolic hormone IGF-1, the growth factor FGF23, and the substrate phosphate, reflect their importance for bone health, it is well established that IGF-1 (as a nutritional marker) is lower in conditions linked to PEW, such as DM and CVD.

Chapter 5

FUTURE PERSPECTIVES

The present thesis attempts to explore clinical correlates and potential determinants of mineral and bone disorders in patients with CKD, and the results indicate important clinical implications of these disorders, in particular for the worsening of survival of patients. As we could confirm that previously described important relations of BMD with both the FGF23 and the IGF-1 systems are of importance in CKD, the interaction between these two systems need to be more explored. Thus, further studies of FGF23 and IGF-1, including if possible also intervention studies, both in animal models and in patients are warranted. Altogether the results of the studies in this thesis may represent steps towards defining and exploring new diagnostic and therapeutic strategies in the CKD population.

Future studies in many fields not explored in this thesis are however needed to better understand both the etiopathology of the vast complex of CKD-MBD and the mechanisms by which these disorders contribute to increased morbidity and mortality among these patients. Several modifiable lifestyle factors such as smoking and alcohol intake as well as factors related to ageing and the altered hormonal situation in CKD patients including decreased levels of testosterone are probably of utmost importance to bone and mineral disorders also in the setting of renal failure. Elderly male smokers have lower concentrations of both 25-hydroxycholecalciferol and intact PTH [164] and develop a decreased levels of testosterone that could predispose to low bone mass [165]. While there at present are few studies on links between lifestyle factors and bone loss in the CKD population, the association between bone loss and food intake is receiving increasing attention. For example, a diet decreasing the urinary excretion of acid ions is thought to be a risk factor for osteoporosis while a diet rich in alkaline content is presumed to be beneficial to bone health [166]. Results of studies designed to test

the potential benefits of alkalinizing salts on bone turnover are however conflicting. Long-term randomized studies were not able confirm a positive influence of alkalinizing salts and dietary calcium intake on the prevention of bone mass loss [167; 168] while a negative association between gastric acid suppression therapy and BMD was reported in HD patients [13]. In line with this negative result, the additional consumption of fruits and vegetables neither reduced bone turnover nor prevented areal BMD decline when compared with placebo group [169]. Based on these evidences and previous studies, in the context of osteoporosis prevention, modification of dietary habits could be a plausible preventive and therapeutic option provided that long-term efficacy could be more clearly demonstrated. On the other hand, because calcium and vitamin D supplementation are in general combined with alkaline salts, the risk of enhancing vascular calcification as a consequence of consumption of food with a high content of alkaline salt should not be ruled out. One study reported that a high acid load with relatively low calcium intake did not appear to accelerate bone loss or increase the risk of fragility-linked fractures [170]. Finally, the Framingham group has reported that there appear to be no relationship between urinary PH or urinary acid excretion and the change of lumbar or femoral BMD [171].

Genetic studies in mineral metabolism are new hot topics. Recently, some studies found several candidate genes regulating bone density in different body sites by gene-based genome-wide association studies (GWAS) technology. For example, through a GWAS meta-analysis approach, the BMD in the spine was found to be regulated by two genes (SP7, meta p-4.4×10^{-6}; C6orf97, meta p-7.7×10^{-7}), and BMD of the femoral bone was found to be linked with two other genes (CKAP5, meta p-5.2×10^{-6}; LRP4, meta p-1.2×10^{-6}); while the genes linked to spine BMD are involved mainly in development of connective tissue, and the skeletal and muscular system, the hip BMD-related genes are implicated in the development and function of the cardiovascular system, tissue morphology, connective tissue system, digestive system and embryonic system [172]. These results underline the importance of the links between bone and other systems. Another study identified SNP rs2273601 in Jagged 1 (JAG1) as being associated with spine BMD in an Asian population [173]. Although there until now have been few studies on the genetic impact on BMD in the CKD population, GWAS and single SNP technology are likely to play a role as novel tools to explore factors involved in bone density and other aspects of bone health in CKD in the future.

Chapter 6

ACKNOWLEDGEMENTS

Acknowledgement is a long word, and although time has passed quickly, the PhD period has been long. I would like to express my sincere gratitude to all who have been involved in this work. This research would not have been possible without the assistance and support of many people who have contributed in various ways to making this thesis come true. In particular, I would like to express my gratitude to:

Bengt Lindholm, my principal supervisor, for giving me this great opportunity to be a PhD student in Baxter Novum and Renal Medicine in this excellent university, guiding me with patience, enthusiasm, encouragement, and excellent scientific stringency throughout the study period, and for mental support when I felt sad and lonely in this foreign country far away from my family. It has been a pleasure to be your doctoral student. I am also thankful for friendship and your career advice, which lightens the prospects of my future work.

Abdul Rashid Qureshi, my co-supervisor, not only for support and inspiring collaboration and supervision and teaching in statistics and for helping me to become familiar with advanced statistical methodology; but also for listening to my complaints, forgiving my moodiness and for protecting me from disappointments in research and life, and for encouraging me to be brave and strong.

Tobias Larsson, my co-supervisor, many thanks for providing me the opportunity to try lab experiments and animal work, for your excellent scientific guidance and the unselfish discussion of research in the PhD study and regarding my career pathway. I

appreciate your great interest in science and the effort you put in trying to understand other peoples point of view.

Peter Stenvinkel, my co-supervisor, for your great expertise in the field of nephrology, for the scientific guidance, for helping me all the time when I needed, for believing in my ability and giving me chances to write review articles, and for sharing all your excellent ideas in the research of renal disease.

Juan Jesus Carrero, my officemate for four years: Although you did not formally supervise me in my PhD project, I appreciate your support and for providing me the opportunity to take part in research in other areas such as insulin resistance, dietary intake and fractures, and also for scientific guidance in clinical epidemiology and for helping me to understand successful thinking, and most importantly, for the positive office environment you made for me.

Tao Wang, my domestic supervisor in China, for guiding me to clinical research and recommending me to this great university and research group, and for making me have chances to touch the joy of science.

Yan Li, my external mentor, for giving me the enlightenment of cell experiments, for sharing your opinions on career options, positive attitude, and for encouraging me all the time.

Bjorn Anderstam, head of Renal Laboratory, and **Monica Ericsson** and **Ann-Christin Bragfors-Helin**, lab technicians at the Clinical Research Center (KFC), lab technicians at the Clinical Research Center (KFC), for kindness and for assisting and supervising me on biomarker assessment and coordination of sample handling. All the **AKM 8 Staff**, for the animal caring.

All study participants and animals, for devoting samples and contributing to my research.

I am also in sincere debt to the administrators at Baxter Novum, **Maj Tofvesson, Titti Lundmark** and **Evianne Larsson**. All of you make my life here feeling like at home and enjoyable.

6. ACKNOWLEDGEMENTS

I am very grateful to my colleagues in the laboratory, **Hannes Olauson, Karolina Lindberg, Risul Amin,** and **Christina Hammarstedt**, for supporting me from the first step in the lab. For all the collaborators that have contributed to this thesis and to friends who have been working or visiting the divisions during my stay: **Elvia Garcia-Lopez, Peter Bárány, Olof Heimbürger, Jacek Waniewski, Thaine Gama Axelsson, Jonas Axelsson, Xiang Hua, Jia Sun, Meng Li, Jiangzi Yuan, Harvest F.Gu, Jose Divino, Mohanmed Suliman, Martin Schalling, Louise Nordfors, Ivan Rodríguez Cabezas, Catalina Martin Cleary, Anna Machowska, Beatriz Fernandez, Silvia Ros, Nisa Buset Ríos, Rafael Jose, Juan Antonio Bravo Soto, Elena Gonzales, Joanna Stachowska-Pietka, Magdalena Jankowska, Tetsu Miyamoto, Tae Yamoto, Ayumu Nakashima, Sawako Kato, Naohito Isoyama, Paul Leurs, Sung-Hee Chung, Sun-Hee Park, Shirley Yumi Hayashi, Marcelo Nascimento, Maria Chan, Karin Edvardsson, Göran Andersson, Annika Wernerson, Yves Sabbagh, Susan Schiavi, Vincent Brandenburg, Markus Ketteler, Fredrik Uhlin, Per Magnusson, Anders Fernström, Qiang Yao** and **David Xinghua Wei**.

All co-authors and colleagues not mentioned above.

I also thank my Chinese collaborators of other ongoing projects, my good friends, **Xiaoyan Huang** and **Hong Xu** for great ideas and excellent discussions on insulin resistance, dietary assessment, nutrition and fractures, and many other topics. I look forward to doing exciting research together with you in the future.

I would also take this opportunity to thank all my Chinese friends: **Chenglin Wu, Wei Xiao, Jian Yan, Peilin Guo, Xiaoyan Liu, Xin Wang, Bin Li, Yimeng Yin, Xiaojing Li, Jia Sun, Tianwei Gu, Chengjun Sun, Ning Wang, Jie Yan, Ying Ye, Rui Wang, Qinzi Yan, Xiaoyuan Zheng, Xiaoying Zhang, Hui Zhang, Yubao Wang, Shuo Huang, Min Wang, Xiaozhen Li, Xiaohui Jia, Na Guan, Yang Xuan, Liang Wei, Cuiqin Li, Yu Jiao, Luan Liu, Jinsong Wang, Na Wang, Miao Zhao, Song Gao, Jiaxue He, Xu He, Tao Han, Yang Li, Ning Xu, Feng Gao, Wei Yu** and **Xiaotian Zhang**, for sharing the happiness, and sometimes the painfulness, with me in Stockholm.

Finally, I express my deepest love to my parents, for supporting me all the time and being the source of the warmest love of my life.

6. ACKNOWLEDGEMENTS

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